MOLECULAR AND CELLULAR BIOLOGY, Apr. 1989, p. 1659–1666
0270-7306/89/041659-08$02.00/0

Vol. 9, No. 4

Molecular Analysis of *GPH1*, the Gene Encoding Glycogen Phosphorylase in *Saccharomyces cerevisiae*

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Received 19 November 1988/Accepted 13 January 1989

In yeast cells, the activity of glycogen phosphorylase is regulated by cyclic AMP-mediated phosphorylation of the enzyme. We have previously cloned the gene for glycogen phosphorylase (*GPH1*) in *Saccharomyces cerevisiae*. To assess the role of glycogen and phosphorylase-catalyzed glycogenolysis in the yeast life cycle, yeast strains lacking a functional *GPH1* gene or containing multiple copies of the gene were constructed. *GPH1* was found not to be an essential gene in yeast cells. Haploid cells disrupted in *GPH1* lacked phosphorylase activity and attained higher levels of intracellular glycogen but otherwise were similar to wild-type cells. Diploid cells homozygous for the disruption were able to sporulate and give rise to viable ascospores. Absence of functional *GPH1* did not impair cells from synthesizing and storing trehalose. Increases in phosphorylase activity of 10- to 40-fold were detected in cells carrying multiple copies of *GPH1*-containing 2μm plasmid. Northern (RNA) analysis indicated that *GPH1* transcription was induced at the late exponential growth phase, almost simultaneous with the onset of intracellular glycogen accumulation. Thus, the low level of glycogen in exponential cells was not primarily maintained through regulating the phosphorylation state of a constitutive amount of phosphorylase. *GPH1* did not appear to be under formal glucose repression, since transcriptional induction occurred well in advance of glucose depletion from the medium.

Energy required for cell activity and growth is maintained in part by regulated synthesis and breakdown of storage carbohydrates. Glycogen phosphorylase, which catalyzes the release of glucose 1-phosphate from glycogen, plays a vital role in this process in many cells and organisms. The regulation of phosphorylase is best understood in vertebrate tissues, in which the enzyme activity is modulated through changing levels of effector ligands and hormonal signals (20). Vertebrate phosphorylases convert between inactive and active forms by reversible covalent modification in the well-characterized regulatory kinase cascade (28). Enzyme activity is also regulated through tissue-specific expression of different isozymic forms. At least three different isozyme forms have been identified in mammals (muscle, liver, brain, or fetal forms, named after the tissues in which they predominate) (19, 43, 54). These isozymes display different allosteric properties, reflecting evolution of their functions to suit specific environments and physiological roles. In the case of the muscle phosphorylase from rabbit skeletal tissue, regulatory mechanisms have been extensively characterized, biochemically and crystallographically (12, 13, 25).

Unlike bacterial and plant phosphorylases, which are unregulated enzymes (8, 33, 35), yeast phosphorylase is also regulated through allosteric mechanisms. The enzyme is subject to secondary-site allosteric control (glucose 6-phosphate inhibition) and is converted between active and inactive forms by reversible phosphorylation (14). An important distinction of yeast phosphorylase is that its phosphorylation site lacks any sequence similarity to the mammalian enzyme counterpart (30). We have previously cloned the phosphorylase gene of the yeast *Saccharomyces cerevisiae* (we refer to the gene as yeast *GPH1*) and derived the primary structure of this enzyme (23). These data, along with recently determined primary structures for bacterial, plant, and isozymic variants of mammalian phosphorylases, have provided a foundation for comparing structure-function and evolutionary relationships among the different enzymes (23, 33a). We have now begun a study of the biochemical and physiological role of glycogen phosphorylase in *S. cerevisiae*, using the powerful tools of genetic manipulations that are available for this organism (45).

Yeast cells contain two principal storage carbohydrates, glycogen and trehalose (15). Both carbohydrates appear to provide energy for cells during periods of nonproliferation. A central question in the study of yeast storage metabolism is why the organism utilizes two different reserve carbohydrates. Only in the case of cells undergoing sporulation or respiratory adaptation does evidence exist for distinct roles (27, 31). A related issue is whether glycogen and trehalose pathways are directly coupled (17, 34, 38). Previous studies have shown that levels of glycogen and trehalose in yeast cells change dramatically in response to a variety of different physiological situations (29, 31, 48). The enzymes involved in synthesizing and breaking down these carbohydrates are regulated through the cyclic AMP (cAMP)-dependent kinase system in yeasts (22, 36, 49, 50, 52, 53). Glycogen and trehalose levels, in fact, have been useful phenotypic indicators for studying various mutants in the *RAS*, adenylate cyclase, and cAMP kinase genes (16, 46, 47). However, perhaps because of the complexity in the regulatory pathway controlling these carbohydrates, it has been difficult to use abnormal carbohydrate levels as a basis for isolating mutants with specific defects in glycogen or trehalose metabolism.

The cloning of yeast *GPH1* permits us to address the above issues by using a combined molecular and genetic approach. We here present a preliminary account of the phenotypic consequences of certain genetic manipulations of *GPH1*. We have disrupted the *GPH1* gene in yeast cells, generating haploid cells with disrupted *GPH1* and diploid cells homozygous for the disruption, and have introduced into cells an increased gene dosage of *GPH1* on 2μm plasmids. We have also examined the expression level of *GPH1* transcripts during growth in rich medium.

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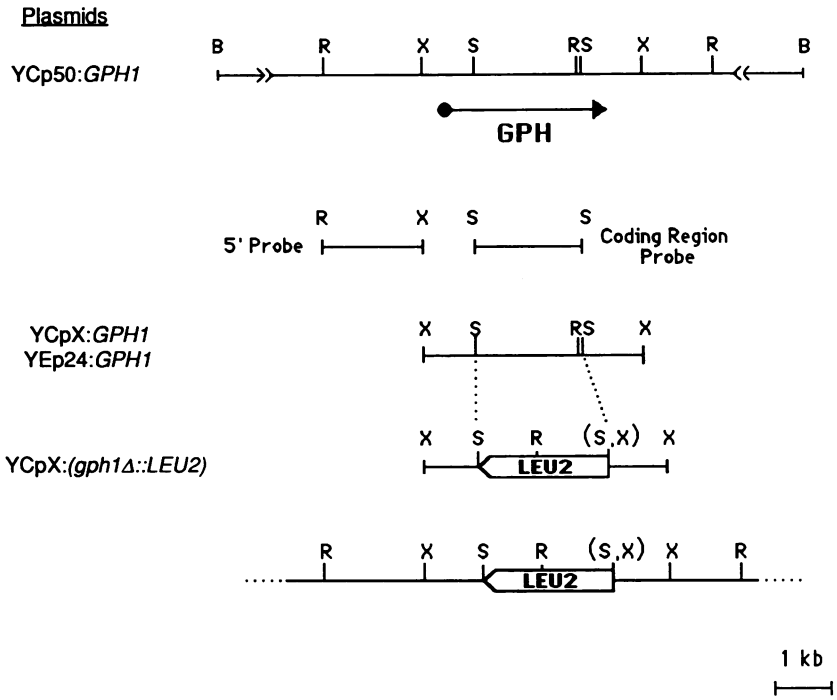

FIG. 1. Structure of wild-type *GPH1*, disrupted *GPH1*, and plasmid constructions. YCp50:*GPH1* contains a 15-kb genomic fragment including the entire *GPH1* gene. The coding region of *GPH1* was previously identified (23). Fragments designated 5′ probe and coding region probe were subcloned into M13 vectors and used as hybridization probes. Plasmids YCpX:*GPH1*, YEp24:*GPH1*, and YCpX:(*gph1*Δ::*LEU2*) were constructed as described in Materials and Methods. Arrow indicates coding region for *GPH1*; open bar indicates yeast *LEU2* gene. Restriction enzyme sites: *Bam*HI (B); *Eco*RI (R); *Sal*I (S); and *Xho*I (X).

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* HR125-5d (*MATa*/*MAT*α, homozygous for *his3 his4 leu2-3 leu2-112 ura3-52 trp1-289*), provided by R. Jensen, was used for RNA and DNA blotting and gene disruption experiments. *Escherichia coli* MH1 (*araD139 lacX74 galU galK hsr rpsL*) and MH6 (*lacX74 galU galK hsr rpsL leuB600 pyrF*::Tn*5*), from M. Hall (18), were used to grow various plasmid constructions.

YM-P medium (31) contained 6.3 g of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) per liter, 4.5 g of yeast extract (Difco) per liter, 9 g of peptone (Difco) per liter, 9 g of succinic acid per liter, 5.4 g of NaOH per liter, and 2% glucose.

Genetic methods. Standard methods of yeast genetics were used for tetrad analysis and scoring of various markers (44). Yeast cells were transformed by the method of Ito et al. (24). Gene disruption was carried out by homologous recombination of a cloned DNA fragment with the genomic DNA, using the one-step gene replacement technique (42).

Plasmids. *GPH1* was originally isolated as a 15-kilobase (kb) genomic DNA insert within the *Bam*HI site of YCp50. This plasmid, designated YCp50:*GPH1*, was used for restriction mapping of the genomic fragment (Fig. 1). The 3.7-kb *Xho*I restriction fragment of *GPH1* was subcloned by digesting YCp50:*GPH1* with *Xho*I and then religating the digested mixture to form the plasmid YCpX:*GPH1*, a ligation of the *GPH1* fragment to an *Xho*I fragment consisting of a portion of YCp50 (*amp*^{r} *ori*) joined to a short region of yeast genomic DNA. For gene disruption, the 1.9-kb *Sal*I restriction fragment within the *GPH1* coding region was removed in YCpX:*GPH1* and replaced by the 2.1-kb *Sal*I-*Xho*I restriction fragment of the yeast *LEU2* gene to give YCpX:(*gph1*Δ::*LEU2*). YEp24:*GPH1* was constructed by inserting the *Xho*I *GPH1* fragment into the *Sal*I site of YEp24.

Preparation of yeast crude extract. Yeast cells grown in liquid cultures were harvested by centrifugation at 3,000 × *g* for 5 min at 5°C. The cell pellets were suspended in cold water, repelleted by centrifugation, and then frozen at −70°C for storage. Thawed pellets were suspended in equal volumes of homogenization buffer (0.1 M Tris [pH 7.9], 10 mM magnesium chloride, 2 mM calcium chloride, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol). Homogenization was carried out by vortexing samples with an equal volume of glass beads (0.5-mm diameter; Biospec Products, Bartlesville, Okla.) in six 30-s pulses. The homogenized mixture was then centrifuged at 8,000 × *g* for 10 min. A portion of supernatant extract was diluted 1:200 for measurement of A_{280}. Typical values were in the optical density range of 0.4 to 0.8.

Phosphorylase assay. The procedure used to assay phosphorylase activity in yeast crude extract is based on the method of Carney et al. (7) and determines the amount of phosphate released during formation of glycogen from glucose 1-phosphate. Enzyme reactions were carried out in 100 mM sodium succinate (pH 5.8)–1% glycogen (prepared from oyster; Sigma Chemical Co., St. Louis, Mo.)–150 mM glucose 1-phosphate–1 mM dithiothreitol. Typically, yeast extracts were diluted 1/100 into the reaction mixtures. To check whether P_i was generated in the assay from other sources within the yeast extract, additional assays were carried out in the absence of added glycogen or glucose 1-phosphate. Portions of reaction mixture were mixed with phosphate-reacting colorimetric reagent (7) and extracted with an equal volume of butanol, which was measured for A_{310}. Phosphate concentration was determined from linear

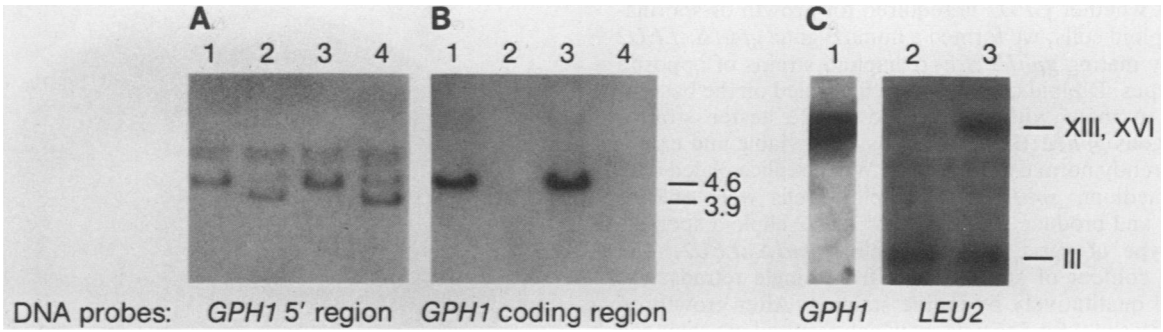

FIG. 2. Disruption and chromosomal localization of the *GPH1* gene. (A and B) Total cellular DNA was prepared from haploid yeast strains segregated from a tetrad derived by the transformation of HR125-5d with the *gph1*Δ::*LEU2* DNA fragment. Then 5 μg of each DNA sample was digested with *Eco*RI, electrophoresed on a 1.0% agarose gel, and transferred to a nitrocellulose filter. Lanes show DNA in Leu^- (1 and 3) and Leu^+ (2 and 4) segregants. (A) Filter hybridized (high stringency) with a ^{32}P-labeled 1.7-kb *Eco*RI-*Xho*I DNA fragment 5′ flanking to *GPH1*; (B) hybridized (low stringency) to a 1.9-kb *Sal*I fragment from the *GPH1* coding region. In panel A, the shift of the predominant signal from a 4.6-kb band (lanes 1 and 3) to a 3.9-kb band (lanes 2 and 4) is as predicted for *gph1*Δ::*LEU2* disruptants. Weaker hybridization bands indicate other homologies in the yeast genome to the 5′-flanking region of *GPH1*. In panel B, lack of low-stringency hybridization in Leu^+ segregants (lanes 2 and 4) confirms both the deletion of the *GPH1* coding region and the absence of multiple phosphorylase genes. (C) Chromosomes from a Leu^- (wild-type *GPH1*) segregant (lanes 1 and 2) and a Leu^+ segregant (lane 3) were separated by OFAGE and blotted to a nylon membrane filter. Lanes: 1, hybridized with a radiolabeled 3.7-kb *Xho*I fragment of *GPH1*; 2 and 3, hybridized with a 2.1-kb *Sal*I-*Xho*I fragment of the yeast *LEU2* gene.

interpolation of spectrophotometrically measured phosphate standards. One unit of phosphorylase activity represents the release of 1 μmol of phosphate from glucose 1-phosphate per min at 30°C. For determination of phosphorylase specific activity, total protein concentration was estimated by the method of Bradford (4), using a bovine serum albumin standard curve.

DNA and RNA hybridization analysis. Total yeast DNA prepared from saturated cultures (11) was digested with *Eco*RI. The DNA was electrophoresed on a 1.0% agarose gel and blotted onto a nitrocellulose membrane filter. DNA probes used for hybridization were gel purified and ^{32}P labeled (10^9 cpm/μg of DNA) by the random primer method (21). Hybridization conditions were 65°C, 6× SSPE (20× SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4, and 20 mM EDTA [pH 7.4]), 5 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Sigma], 5× Denhardt solution, 10 μg of salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate. Washes were carried out nonstringently in 2× SSPE or stringently in 0.1× SSPE in the presence of 0.1% sodium dodecyl sulfate at 65°C.

For chromosomal analysis of *GPH1*, yeast chromosomes were separated by orthogonal-field alternating-gel electrophoresis (OFAGE) on a 1.5% agarose gel (6). The gel was blotted onto a nylon membrane filter and UV irradiated (9). The filter was hybridized to ^{32}P-labeled DNA probes as described above.

Total cellular RNA was isolated as described by Jensen et al. (26). RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane filter with UV fixation (9), and hybridized under stringent conditions (42°C, 50% formamide, 5× SSPE, 5× Denhardt solution, 0.1% sodium dodecyl sulfate) to the ^{32}P-labeled 1.9-kb *Sal*I fragment of *GPH1*.

Cellular and medium parameters. For growth curve monitoring, liquid growth was carried out in 500-ml baffled Erlenmeyer flasks (type 2543; Bellco Glass, Inc., Vineland, N.J.), each containing 100 ml of medium, and incubated in a rotary shaker at 200 rpm and 30°C. Cell density was monitored primarily by A_{600} after a calibration of optical density to cell number was determined. Initial determinations of cell number and percentage of budded cells were made by using a cell-counting chamber (Hausser Scientific, Philadelphia, Pa.). Medium glucose levels were determined by using a glucose oxidase kit from Sigma.

Determination of glycogen and trehalose content. Qualitative glycogen determinations were made by adding 5 to 10 ml of a 0.4% KI–0.2% iodine solution onto plates. Cells that accumulate glycogen stain brown, whereas those that do not remain yellow. Quantitative determinations of glycogen and trehalose were carried out by methods described by Lillie and Pringle (31).

RESULTS

Construction of a null *GPH1* allele. To determine whether *GPH1* is essential to haploid cell growth, a *gph1*Δ::*LEU2* allele was constructed by the method of Rothstein (42). On a cloned DNA fragment containing the *GPH1* gene, a 1.9-kb *Sal*I restriction fragment within the coding region was deleted and replaced by an insertion of a *LEU2* gene fragment (Fig. 1). The resulting plasmid, YCpX:(*gph1*Δ::*LEU2*), was cut with *Xho*I, and the gel-purified 3.8-kb *gph1*Δ::*LEU2* DNA fragment was used to transform the diploid HR125-5d to leucine prototrophy. Three Leu^+ diploids were sporulated, and tetrads were analyzed. In 13 of 16 tetrads (5 of 5, 4 of 5, and 4 of 6), all spores were viable, with 2:2 segregation of Leu^+:Leu^-, and grew at similar rates.

DNA was prepared from four spore colonies derived from a single tetrad, and Southern hybridization analysis was used to confirm that *GPH1* was replaced by the disrupted gene in the two Leu^+haploids (see Fig. 2A and B). In addition, chromosomes from Leu^- and Leu^+ haploids were separated by OFAGE (6), blotted to filters, and hybridized to *GPH1* and *LEU2* probes (Fig. 2C). A preliminary assignment of *GPH1* to chromosome XIII or XVI was made by detection of *GPH1* hybridization to band 10 of the OFAGE separation (6). The *LEU2* probe hybridized to this same band only in Leu^+ cells, providing additional confirmation of correct gene disruption. The absence of low-stringency hybridization in Leu^+ disruptants to the 1.9-kb DNA encoding the catalytic portions of yeast phosphorylase (Fig. 2) indicated that haploid cells contained no other copy of a phosphorylase gene. Therefore, since *GPH1* and *gph1*Δ::*LEU2* segregants were equally viable, we conclude that *GPH1* was not essential to vegetative growth.

To test whether *GPH1* is required for growth or sporulation in diploid cells, we formed a homozygous *gph1*Δ::*LEU2* diploid by mating *gph1*Δ::*LEU2* haploid strains of opposite mating types. Diploid colonies were identified on the basis of inability to mate with *MAT***a** and *MAT*α tester strains. Homozygous *gph1*Δ::*LEU2* diploids were viable and exhibited apparently normal morphology. When replica plated onto acetate medium, *gph1*Δ::*LEU2* diploid cells were able to sporulate and produce viable *gph1*Δ::*LEU2* haploid spores.

Phenotype of yeast cells containing *gph1*Δ::*LEU2*. The glycogen content of spore clones from single tetrads was examined qualitatively by iodine staining. After growth on YEPD plates (44) for 5 to 7 days, which resulted in prolonged incubation of cells at stationary phase, spore colonies began to show differences in staining intensity for glycogen (Fig. 3) that depended on whether the *GPH1* gene was disrupted. Darker and lighter iodine staining tended to develop in an expected 2:2 ratio in tetrad spores. Higher intracellular glycogen content, which is indicative of reduced glycogen breakdown, segregated with disruption of *GPH1* and leucine prototrophy.

A more direct comparison of *GPH1* function in *gph1*Δ::*LEU2* and *GPH1* cells was obtained from assays of phosphorylase activity in homogenized crude extracts of yeast cultures grown to stationary phase (Table 1). The assay procedure monitors the release of P_i as phosphorylase catalyzes the incorporation of glucose 1-phosphate into glycogen. The Leu$^+$ segregants released significantly less phosphate than did Leu$^-$ cells, which indicated the absence of phosphorylase activity. The phosphate released from *gph1*Δ::*LEU2* cells (PH3-6b and PH3-6d) most likely resulted from other phosphate-generating activity in the cell extract. Some measure of this contribution was obtained from assay reactions omitting phosphorylase substrates glucose 1-phosphate and glycogen (Table 1, last two columns). In the results of assays without glycogen, it should be noted that glycogen from the diluted cell extract may still promote a significant degree of phosphorylase-catalyzed phosphate release.

Experiments were carried out to determine other phenotypic differences between Gph$^+$ (*GPH1*) and Gph$^-$ (*gph1*Δ::*LEU2*) cells. Wild-type and mutant strains were grown under identical conditions and monitored for various medium and cellular parameters. YM-P (2% glucose) was used for growing cultures, since yeast growth and carbohydrate accumulation had previously been characterized in this medium (31). Overall, the Gph$^+$ and Gph$^-$ strains exhibited similar growth profiles (see Fig. 4A). Doubling times during exponential growth were identical. The consumption of medium glucose followed similar time courses. Adaptation from fermentative to respirative growth and entry into stationary phase also appeared similar between Gph$^+$ and Gph$^-$ strains. Cell morphologies, as examined microscopically, were similar throughout the growth curve; the percentage of budded cells dropped in the same manner as both strains reached stationary phase.

Intracellular glycogen and trehalose contents were also determined during the growth of Gph$^+$ and Gph$^-$ cells (Fig. 4). In wild-type yeast cells, levels of these carbohydrates are generally low but increase significantly as cells approach growth-arresting conditions (31). (Measurements of glycogen and trehalose may vary widely depending on the medium and strain used, the method of normalization, and perhaps factors of imprecision intrinsic to the assay procedures.) Both Gph$^-$ and Gph$^+$ cells exhibited the wild-type pattern of carbohydrate accumulation. In particular, glycogen accumulation in both strains began at similar moments, near the end of exponential growth, but well in advance of glucose exhaustion in the medium. Although the basal level of glycogen in exponential Gph$^-$ cells appeared to be somewhat higher than in Gph$^+$ cells, the general similarity of glycogen profiles in the presence or absence of phosphorylase indicated that phosphorylase was not the principal regulator of glycogen content during proliferative growth. During stationary phase, Gph$^-$ cells contained significantly more glycogen than did Gph$^+$ cells, understandably because of the absence of phosphorylase-catalyzed degradation in the Gph$^-$ cells. It has been suggested that glycogen may be used for respirative adaptation during diauxic growth (31). However, there was no indication in our studies that such adaptation and consequent entry into stationary phase were affected by loss of phosphorylase function.

Comparison of trehalose levels in the Gph$^+$ and Gph$^-$ cells enabled us to determine whether absence of phosphorylase-catalyzed glycogenolysis affects the pathway of

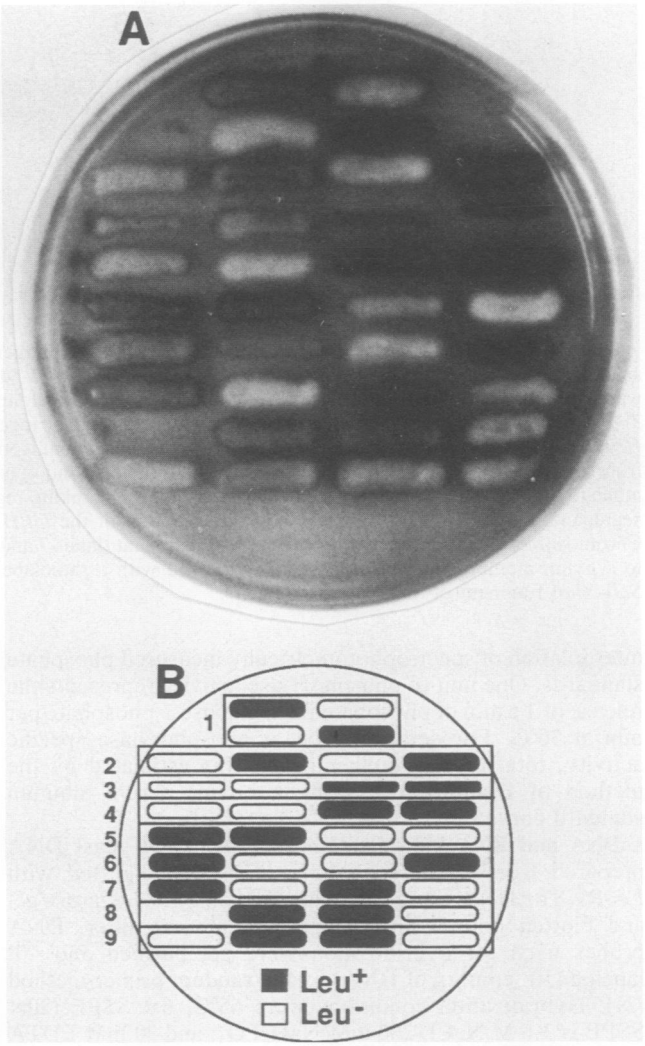

FIG. 3. Glycogen staining of haploid progeny derived from *GPH1*-disrupted diploid cells. (A) Staining of transformed HR125-5d cells. Cells were sporulated, and tetrads were dissected by micromanipulation. Segregants from nine tetrads were germinated on YEPD medium (44) at 30°C for 2 days and then streaked onto the YEPD plate shown, grown for 5 days, and stained with a solution of 0.4% KI–0.2% I_2. (B) Diagrammatic representation of plate showing the grouping of spores derived from individual asci and the segregation pattern of leucine prototrophy. In tetrad 8, one of the four spores (a *GPH1* segregant) was not viable.

TABLE 1. Phosphorylase activity in spore clones derived from a *GPH1*-disrupted transformant[a]

Strain	*MAT*	Leucine prototrophy	Glycogen[b] accumulation	Phosphate release (nmol/min per mg of total protein)[c]		
				Complete reaction	Without added glucose 1-phosphate	Without added glycogen
PH3-6a	a	−	−	28.0	6.7	12.2
PH3-6b	a	+	+	6.4	4.1	9.3
PH3-6c	α	−	−	19.0	3.0	11.7
PH3-6d	α	+	+	9.3	2.8	9.1
PH3-6b/*GPH1*-2μm[d]	a	+	−	573.0		
PH3-6d/*GPH1*-2μm[d]	α	+	−	373.0		

[a] Strains PH3-6a, PH3-6b, PH3-6c, and PH3-6d represent a single tetrad derived from sporulation of *GPH1*-disrupted HR125-5d cells. Cell cultures were grown to early stationary phase in liquid medium before preparation of crude cell extract for the enzyme assay.
[b] Detected qualitatively by iodine staining.
[c] Equivalent to milliunits of phosphorylase activity per milligram of protein.
[d] *GPH1* present on multicopy plasmid YEp24.

trehalose synthesis. Gph$^+$ and Gph$^-$ cells contained similar amounts of trehalose during exponential growth and at stationary phase. During mid- to late log growth, both strains exhibited slightly decreasing levels of trehalose. At the end of exponential growth, Gph$^+$ and Gph$^-$ cells showed a similar pattern of trehalose accumulation. As has been observed in other wild-type yeast strains (31), trehalose synthesis in Gph$^+$ and Gph$^-$ strains lagged behind the initiation of glycogen accumulation but continued after cells had reached stationary phase. The absence of functional *GPH1* did not impair the capacity of cells to synthesize and store trehalose, nor did it alter the temporal relationship of trehalose and glycogen accumulation.

Since glycogen in yeast cells is normally degraded during stationary phase, we expected that *GPH1* and *gph1*Δ::*LEU2* cells might exhibit differences in cell viability after incubation at stationary phase. However, we observed no difference when *GPH1* and *gph1*Δ::*LEU2* cultures were incubated in exhausted YM-P medium for 4 weeks. Although neither yeast strain showed detectable loss of viability, it is possible that longer incubation may be required for a difference to be detected. In wild-type yeast strains, net degradation of glycogen or trehalose is undetectable until after 2 weeks; furthermore, cell viability fails to decline during 3 months of incubation at room temperature (31).

An attempt to discover a condition under which *GPH1* confers an advantage to yeast cells was made by challenging *gph1*Δ::*LEU2* and *GPH1* cells to grow at 36°C. Gph$^+$ and Gph$^-$ cells were plated onto YEPD medium and grown at 36°C. Again, however, no difference in cell viability or growth rate was found between *GPH1* and *gph1*Δ::*LEU2* yeast strains.

Increased gene dosage of *GPH1* through integration onto plasmid YEp24. The phenotypic consequence of an increased dosage of *GPH1* was determined by transfecting yeast cells with a multicopy plasmid containing the *GPH1* gene. YEp24:*GPH1*, constructed as described in Materials and Methods, consisted of a 3.7-kb *GPH1* fragment inserted into the 2μm plasmid YEp24 (*URA3*). YEp24:*GPH1* was used to transform PH3-6b and PH3-6d strains. Ura$^+$ transformants from either strain were found to contain levels of phosphorylase activity 10- to 40-fold higher than wild-type levels (Table 1). Using strains with a disabled genomic copy of *GPH1* assured us that phosphorylase overproduction involved transcription from the plasmid-derived *GPH1* gene and that the gene fragment cloned into the plasmid did not lack any *cis*-acting element that might be required for *GPH1* induction. Transformed cells were found to contain less glycogen than wild-type cells, as determined by iodine staining and glycogen assay (data not shown). Apart from the differences in phosphorylase activity and glycogen content, transformants appeared physiologically and morphologically similar to the untransformed strains. In particular, no differences in the growth profile or in the accumulation pattern of glycogen were detected. Furthermore, there was no difference between transformed and wild-type strains in cell viability after a 2-week incubation at stationary phase.

Growth cycle dependence of *GPH1* mRNA expression. Total RNA was prepared from wild-type cells harvested at several time points during growth in YM-P medium. Various medium and cellular parameters were also monitored at harvesting points (Fig. 5B). A band of RNA corresponding to a 3.3-kb RNA species was detected by radiolabeled *GPH1* probe in samples from late exponential and stationary cells. *GPH1* transcripts were not observed in the initial growing cells, an indication that the *GPH1* gene is repressed during exponential growth. Interestingly, the highest level of *GPH1* transcript was detected in cells that were still proliferating exponentially while medium glucose was slightly declining. This time point also corresponded to the onset of glycogen accumulation (Fig. 4). The *GPH1* gene did not appear to be under strict glucose repression, since peak levels of transcripts occurred well in advance of glucose exhaustion from the medium. Glycogen accumulation has previously been shown to occur before the depletion of glucose from medium (31). The coincidence of the initial appearance of *GPH1* transcripts and glycogen accumulation suggests coordinated regulation of glycogen phosphorylase and glycogen synthase genes.

It should be noted that detected *GPH1* transcripts were not normalized to the mRNA of a constitutively expressed gene, as is commonly done in Northern (RNA) analysis profiles. This could not be done in our study since yeast cells generate quite different mRNA profiles between logarithmic and stationary phases (3). By loading as close to equal amounts of total RNA as possible in all gel lanes, we in effect achieved normalization to the rRNA species that predominate in total RNA preparations.

As an extension of the experiment described above, *GPH1* transcripts were monitored in yeast cells during stationary-to-log-phase adaptation. Cells were initially grown to saturation in YM-P and incubated for 6 days in the exhausted medium, after which they were pelleted and suspended in fresh medium. *GPH1* mRNA gradually diminished to undetectable levels, although transcripts were still present in cells after two doublings during the preexponential phase (data not shown).

DISCUSSION

Yeast phosphorylase is activated allosterically through reversible phosphorylation at a specific threonine residue

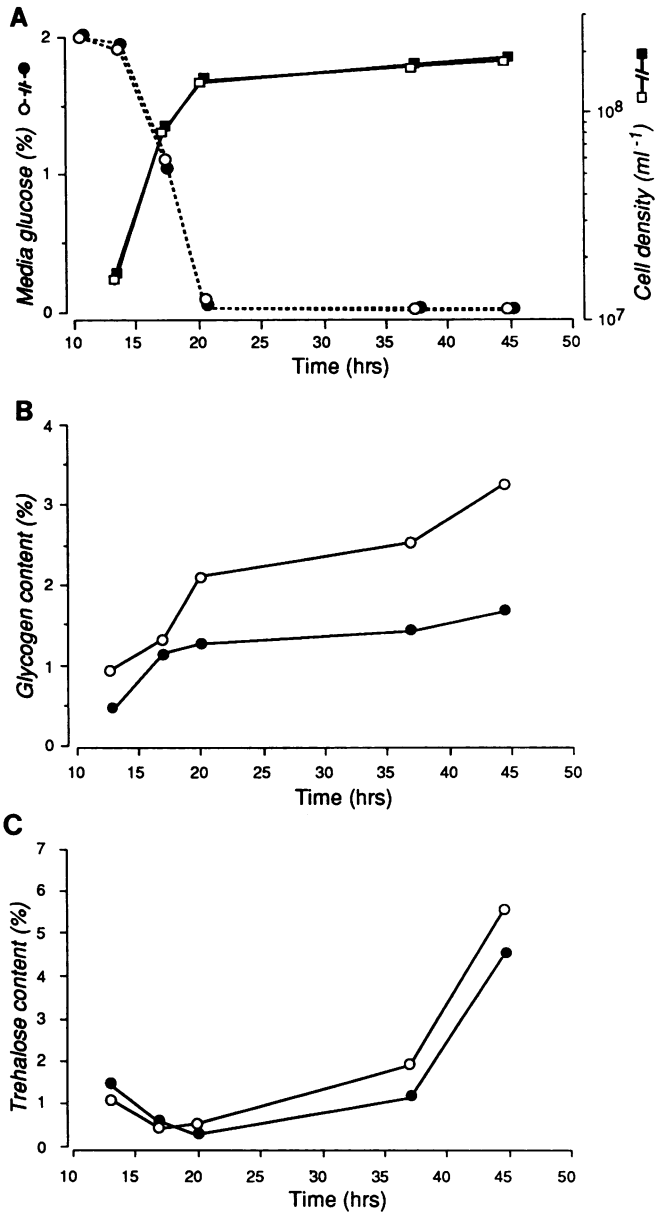

FIG. 4. Growth profiles (A), glycogen accumulation (B), and trehalose accumulation (C) of Gph^+ and Gph^- yeast cells approaching stationary phase in a rich medium. YM-P medium containing 2% glucose was inoculated (at $t = 0$ h) with exponentially growing cells from YM-P precultures to give initial cell densities of 10^4/ml. The cultures were then incubated at 30°C in a rotary shaker at 200 rpm. Sampling was begun when the cell density reached approximately 10^7/ml. Symbols: ○, □, Gph^- cells (PH3-6b, a *MAT***a** *gph1*Δ::*LEU2* strain derived from HR125-5d); ●, ■, Gph^+ cells (PH3-6a, a *MAT***a** *GPH1* derivative of HR125-5d).

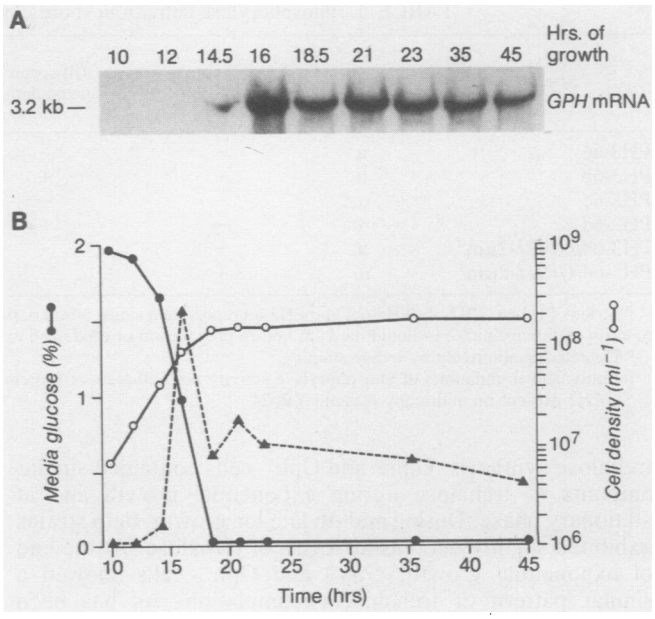

FIG. 5. Time course of *GPH1* mRNA accumulation by yeast cells during the approach to stationary phase in a rich medium. (A) Time course Northern blotting analysis of *GPH1* mRNA levels. Total cellular RNA was isolated from yeast cells growing in YM-P medium containing 2% glucose. Conditions were the same as for the experiment shown in Fig. 4. A 20-μg amount of each RNA preparation was electrophoresed on a 1.2% agarose gel containing formaldehyde and hybridized with the ^{32}P-labeled *GPH1 SalI* fragment. (B) Accumulation of *GPH1* mRNA by yeast cells in relation to glucose concentration and rate of cell proliferation during the approach to stationary phase. The dashed line indicates relative cellular levels of *GPH1* transcript as determined from densitometer scanning of the Northern blot.

and inhibited allosterically through glucose 6-phosphate binding. Phosphorylation is catalyzed by a specific phosphorylase kinase and perhaps additionally by a cAMP-dependent protein kinase (39, 53). The unphosphorylated, less active enzyme form, phosphorylase *b*, is potently inhibited by glucose 6-phosphate, whereas the phosphorylated enzyme, phosphorylase *a*, is less sensitive to this effector. In addition to allosteric controls, yeast phosphorylase is subject to transcriptional regulation. This study indicates that *GPH1* transcript levels are low during exponential growth but increase before entry into stationary phase. Previous immunological evidence has indicated that phosphorylase production in *Saccharomyces carlsbergensis* also occurs specifically at the late exponential growth phase (1). We have observed that intracellular levels of phosphorylase are highest in yeast cells grown to stationary phase (unpublished findings).

One of our objectives is to understand the physiological role of the allosteric regulatory mechanisms in yeast phosphorylase. Our findings refute a model of glycogen metabolism in which a constitutive level of phosphorylase participates in regulating glycogen content during exponential growth through phosphorylation-dephosphorylation interconversion mediated by the cAMP regulatory cascade. Although further study is needed to define the role of allosteric regulation in yeast phosphorylase, current information about carbohydrate metabolism and cAMP regulation in yeasts suggests a reasonable model. As phosphorylase and glycogen accumulate at the end of exponential growth, the enzyme most likely exists in the unphosphorylated *b* form. A predominance of phosphorylase *b* over phosphorylase *a* is consistent with observed decreasing concentrations of cAMP and decreasing activity of cAMP-dependent kinase as haploid yeast cells approach stationary conditions (17, 51). Although less active than the *a* enzyme, phosphorylase *b* has enzymatic activity (14) that may be sufficient to meet a minimal energy demand in stationary cells and to account for a gradual diminution of intracellular glycogen content (31). Degradation of glycogen during stationary incubation would be effectively modulated by intracellular levels of glucose 6-phosphate, which, as already noted, is a more effective inhibitor of phosphorylase *b*. Interestingly, the level of

glucose 6-phosphate in proliferating yeast cells rises as medium glucose is exhausted and then drops back to a lower level when cells are in stationary phase (41).

When stationary yeast cells are switched to rich growth conditions, glycogen levels are rapidly reduced (2), suggestive of a more profound activation of phosphorylase activity, as would be afforded by phosphorylation. Hence, covalent modification of phosphorylase involving the cAMP-mediated activation of an accessory kinase most likely occurs during adaptation to rich medium. Such conditions have been shown to promote increases in cAMP levels and cAMP-dependent kinase activity in yeasts (17, 32, 40, 51). After glycogen depletion, subsequent exponential growth obviates any metabolic requirement to maintain phosphorylase in the cell.

The adaptive value of maintaining two different reserve carbohydrates in yeast cells remains unclear to us. Glycogen and trehalose display nonidentical patterns of accumulation and utilization under varying nutritional limitations (31), which indicates that these reserves may play distinct roles in the cell life cycle. To assess the role of glycogen and phosphorylase-catalyzed glycogenolysis in the yeast life cycle, we have constructed strains lacking a functional phosphorylase gene or containing multiple copies of the gene. *GPH1* does not appear to be an essential gene. Haploid cells disrupted in *GPH1* contained higher levels of intracellular glycogen but otherwise appeared similar to haploid Gph^+ cells. Increasing the gene dosage of *GPH1* by presenting it on a YEp24-based plasmid resulted in an expected increase of phosphorylase activity yet was unaccompanied by any adverse phenotype. The latter high-copy construction enables us to purify phosphorylase from saturated yeast cultures for further biochemical and structural study (V. L. Rath, P. K. Hwang, and R. J. Fletterick, manuscript in preparation).

Since phosphorylase is a principal enzyme in glycogenolysis, the apparent dispensability of *GPH1* suggests that glycogen degradation in yeasts may not be an essential function. Phosphorolysis, however, is not the only mode of glycogen breakdown in yeasts. Cells undergoing sporulation exhibit a period of glycogen degradation during the final maturation of ascospores (27) as a result of the activity of a sporulation-specific glucoamylase encoded by the *SGA* gene (10, 55); glycogen phosphorylase, in fact, is undetectable in yeast cells during sporulation (10). Interestingly, the recent studies in which the *SGA* gene was cloned and disrupted indicate that this gene is not required for successful sporulation (55). This result provides a supporting indication that glycogen degradation may not be essential.

Given the elaborate allosteric properties of yeast phosphorylase and the growth-dependent regulation of *GPH1* transcription, it is difficult to accept dispensability of *GPH1* function. The coincidence of *GPH1* induction and glycogen accumulation at the end of logarithmic growth supports a primary physiological role for phosphorylase in utilizing glycogen as a reserve energy source during periods of nutrient starvation. The high degree of structural and functional conservation between yeast and mammalian phosphorylases further argues that the yeast enzyme, like its mammalian counterparts, serves an essential function in some particular distress. It is possible that the enzyme confers to yeast cells adaptive or growth advantages that are not apparent in homogeneous cultures or under the growth conditions used in the laboratory.

Several reports have suggested that in yeasts, trehalose forms at the expense of glycogen (17, 34). It is evident from our study that disruption of *GPH1* does not impair the capacity of yeast cells to accumulate a high level of trehalose. Therefore, unless an alternative pathway of glycogenolysis functions in the absence of *GPH1*, glycogen degradation does not appear to play an important role in supplying glucose for trehalose synthesis. Since we have observed that trehalose accumulation continues well after the exhaustion of medium glucose, gluconeogenesis is the probable primary source of glucose for trehalose synthesis. Supporting this view is the observation that when nonproliferating yeast cells are incubated in the presence of alanine, a nonfermentable carbon source, the trehalose concentration increases as the amino acid is rapidly depleted (36), an indication that gluconeogenesis provides the precursors of trehalose synthesis.

The most likely explanation for the apparent dispensability of phosphorylase is that under the growth conditions used in this study, utilization of trehalose as the energy source sufficiently compensated for the Gph^- defect. To further examine this matter, we would like to determine stationary conditions that promote metabolism of one or the other carbohydrate but not both. As an alternate approach to dissecting the metabolic roles of glycogen and trehalose, it may be possible to isolate cells that cannot survive without a functional copy of the *GPH1* gene. Since glycogen and trehalose appear to serve overlapping purposes, a mutant search by this approach may identify defective genes specific to the trehalose pathway. Another hope is to identify the gene or genes for yeast glycogen synthase, the enzyme that catalyzes glycogen formation, through possible homology to recently determined DNA or protein sequences of human glycogen synthase (5). Genetic control over both synthetic and degradative pathways of glycogen metabolism will provide the ultimate tools for defining the role of glycogen in the yeast organism.

ACKNOWLEDGMENTS

We are indebted to Michael Hall and Susan Lillie for technical advice and discussions and to Ira Herskowitz for the use of laboratory facilities.

This work was supported by National Science Foundation grant DMB84-20489.

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